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Clinical Significance of Hepatitis C Viral RNA Status and Its Correlation to Antibodies to Structural HCV Antigens in Anti-HCV Reactive Patients With Normal Liver Tests

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Extensive serological testing and HCV RNA determination by RT-PCR was performed in serum, PBMCs, and liver tissue in thirteen anti-HCV reactive patients with persistently normal liver tests. Absolute concordance in the status of HCV RNA between serum, PBMCs, and liver was noted. Five patients were HCV RNA positive but only three had mild histological changes. Eight patients were HCV RNA negative in all three sites and had virtually normal liver histology. Patterns of reactivity in RIBA™2.0 strip immunoblot assay did not differentiate viremic from nonviremic patients. ELISA testing using multiple individual HCV recombinant antigens from the structural and non-structural regions of HCV demonstrated mean antibody titers to the structural antigens, in particular HCV E2 antibodies, to be significantly lower in HCV RNA negative patients. The status of HCV RNA in the serum appears to infer the status of HCV RNA in the liver and PBMCs in patients with persistently normal liver tests. Patients with persistently normal liver tests and undetectable HCV RNA have probably spontaneously cleared HCV infection.

KEY WORDS: HCV infection, asymptomatic, normal aminotransferase levels, hepatitis C virus, HCV E2 antibody

INTRODUCTION

It has been demonstrated that confirmed anti-HCV reactive blood donors with normal ALT, may or may not have detectable HCV RNA in serum [Villa et al., 1991; Alberti et al., 1992; Brillanti et al., 1993; Navas et al., 1993; McGuinness et al., 1993; Bruno et al., 1994; Naito et al., 1994; Wang et al., 1994; Prieto et al., 1995; Shakil et al., 1995; Shindo et al., 1995b]. The presence of HCV RNA in the serum is often associated with some degree of hepatic necro-inflammatory activity [Alberti et al., 1992; Navas et al., 1993; Prieto et al., 1995; Shakil et al., 1995; Shindo et al., 1995b]. Anti-HCV reactive patients with persistently normal liver tests, but without detectable HCV RNA in the serum, may have occult chronic hepatitis C infection, or have recovered from previous hepatitis C infection. We undertook this study to determine the clinical significance of anti-HCV antibodies in patients with persistently normal liver tests; some of whom had detectable HCV RNA in serum and some who did not. A comprehensive evaluation including liver biopsy and HCV RNA determination in serum, liver, and PBMC was undertaken. Extensive serologic testing was also performed in these patients to compare antibody profiles in patients with ongoing infection to those patients without detectable HCV RNA.

SUBJECTS AND METHODS

Patients

Adult patients with confirmed anti-HCV reactivity and persistently normal liver tests for at least 6 months of follow-up at the Los Angeles County-University of Southern California Hepatitis Clinic were eligible for this study. Patients with HBsAg or anti-HIV in serum were excluded. Patients who had consumed more than 80 g alcohol/day for over 5 years and patients with physical evidence of chronic liver disease were also excluded. Between June 1993 and June 1994, 16 eligible patients were seen. Three patients declined to undergo a liver biopsy. The remaining 13 patients constitute the basis of the first part of this study. All patients gave written informed consent for this study, which had been ap-

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proved by the institutional review committee. Patients were evaluated by history, physical examination, anti-HCV analysis, PBMC isolation, and percutaneous liver biopsy. These patients had multiple determinations of a panel of liver tests (including alkaline phosphatase, globulin, albumin, total bilirubin, AST, ALT, prothombin activity) and HCV RNA in the serum (mean 6.7, range 5–17) during a period of clinical observation (mean 14.5 months, range 6–29).

In addition, anti-HCV serologic analysis and HCV RNA testing were performed on serum specimens from eleven presumably healthy blood donors who were confirmed positive for anti-HCV with normal ALT levels.

Methods

All specimens were tested for anti-HCV reactivity by Ortho HCV 2.0 ELISA Test System (ELISA 2)(Ortho Diagnostics, Raritan, NJ), which detects antibodies reactive with c100-3, c200, and c22-3 recombinant antigens. The c200 antigens is an expression product of the putative NS3/NS4 regions of the HCV genome, encoding both the c100-3 and c33c antigens. The c22-3 recombinant protein is an expression product of the core region of the HCV genome. Specimens were also tested with the HCV RIBA™ 2.0 Test System (Chiron Corporation, Emeryville, CA). This strip immunoblot assay (SIA) detects antibodies reactive with HCV antigens 5-1-1, c100-3, c33c, and c22-3. All ELISA 2 and RIBA™ 2.0 testing was carried according to the manufacturer's recommendations.

Titers of antibodies reactive with individual HCV antigens were quantitatively assessed using individual antigen ELISAs. For these ELISAs, HCV recombinant antigens c100-3, c33c, c22-3, and E2 were separately coated on to microwells. E2 is a glycosylated antigen derived from the putative envelope region of the HCV genome and was expressed in Chinese Hamster ovary (CHO) cells [Lesniewski et al., 1995]. Individual antigen ELISAs were carried out exactly as described for ELISA 2. Cutoffs for each ELISA were established by testing 180 specimens from individuals who were at low risk for HCV infection and who did not react in ELISA 2. A cutoff optical density value was selected which was typically greater than 10 standard deviations above the population mean. Serial dilutions of each specimen were tested (in duplicate) by each individual antigen ELISA and end point titers deterined in each case.

Liver biopsy and PBMC samples were obtained from 13 patients. A portion of the liver biopsy was immediately frozen in liquid nitrogen and stored at −70°C until use. PBMCs were separated from 8 ml whole blood collected in VACUTAINER CPT™ tubes with Sodium Citrate (Bectin Dickinson, Franklin Lakes, NJ), then washed eight times with media.

Total RNA was extracted from 20–30 μg liver, 10^7 PBMC, 100 μl serum and the eighth washing of PBMCs, and assayed for HCV RNA by means of reverse transcription-polymerase chain reaction (RT-PCR) using "nested" primers from the 5′ noncoding region of the HCV genome as previously described [Cristiano et al., 1991]. All serum, PBMC and liver samples were extracted in parallel with negative and positive controls. To exclude carryover contamination, for each sample, RT-PCR was carried out without the presence of reverse transcriptase. The sensitivity of the RT-PCR assay was determined by testing known standards from the Quantiplex™HCV RNA Assay (Chiron Corp.). The RT-PCR assay could detect approximately 100 copies of HCV RNA/100 μl serum. To ensure integrity of extracted RNA from liver and PBMC samples, reverse transcription was also carried out using oligo-dT primers. Amplification of liver and PBMCs cDNA was carried out using actin and CD3 primers respectively, as previously described [Barnes et al., 1992].

Each liver biopsy was scored for the degrees of periportal necrosis, intralobular degeneration, portal inflammation, and fibrosis as outlined according to the histologic activity index (HAI) [Knodell et al., 1981]. The pathologist (S.G.) was blinded to the results of HCV RNA determination.

Statistical Methods

The components of the HAI scores of the HCV RNA positive and HCV RNA negative patients undergoing liver biopsy were compared and analyzed using Kruskal-Wallis one way ANOVA and multivariate analysis. The reciprocal antibody titers of HCV RNA positive and negative patients were analysed using two tailed Student *t*-test.

RESULTS

The 13 patients who underwent evaluation and liver biopsy included seven men and six women who ranged in age from 20 to 54 years (mean = 39); eight were white, three were black, and two were Hispanic. Risk factors for HCV infection included intravenous drug use in eight patients, blood transfusion in two patients, and needlestick injury in one patient. Two patients had no parenteral risk factors. The period of parenteral exposure for the 11 patients in relation to the time of their evaluation in this study, is shown in Table I. All 13 patients were asymptomatic. Nine were found to be anti-HCV reactive when donating blood or selling plasma commercially, while four were identified by routine testing for drug rehabilitation programs.

We observed complete correlation between results of HCV RNA testing of serum, PBMCs, and liver tissue. In eight patients, HCV RNA was consistently not detectable in the serum during the observation period. In these patients, HCV RNA was not detectable in PBMC or liver tissue. On the other hand, in the five patients who had persistently detectable HCV RNA in the serum, HCV RNA was detected in their PBMCs and liver.

Liver histology was considered to be essentially normal in all but one HCV RNA negative patients and in two HCV RNA positive patients. However, even among the remaining HCV RNA positive patients, the histological changes were mild. There was significantly less periportal necrosis in HCV RNA negative patients than HCV RNA positive patients ($P = .002$). There was consider-

TABLE I. Demographic Characteristics of HCV Reactive Patients With Normal Liver Tests*

Patient	Age (years)	Sex	Risk	Years at risk[a]
HCV-RNA Negative				
1	41	F	IVDA	22–20
2	31	F	IVDA	14–10
3	42	F	needle stick	14–10
4	32	M	IVDA	5–2
5	38	M	NOS	
6	40	M	IVDA	12–1
7	51	M	NOS	
8	30	M	IVDA	14–2
HCV-RNA Positive				
1	48	F	PT	14
2	44	F	IVDA	25–4
3	30	M	IVDA	10–5
4	29	F	IVDA	15–11
5	54	M	PT	5

*F = female, M = male, IVDA = intravenous drug abuse, NOS = no source, PT = post transfusion.
[a]Period of parenteral exposure prior to evaluation.

TABLE II. Histological Characteristics of HCV Reactive Patients With Normal Liver Tests

	Liver histological activity index (HAI) scores				
Patient	Bridging necrosis (0–10)	Intralobular degeneration (0–4)	Portal inflammation (0–4)	Fibrosis (0–4)	HAI (0–22)
HCV-RNA Negative					
1	0	0	0	0	0
2	0	1	1	0	2
3	0	1	1	0	1
4	0	1	1	0	2
5	0	1	1	0	2
6	1	1	1	1	4
7	0	1	1	0	2
8	0	1	0	0	1
HCV-RNA Positive					
1	3	1	3	1	8
2	1	0	1	0	2
3	3	1	3	1	8
4	1	1	1	0	3
5	3	1	3	1	8

ably less portal inflammation in HCV RNA negative patients than HCV RNA positive patients ($P = .016$). The degree of fibrosis was similar in both groups ($P = .083$). The mean HAI score was also lower in the HCV RNA negative group compared to the HCV RNA positive group ($P = .015$). The results of the histological findings are shown in Table II.

All patient specimens were reactive by ELISA 2 and positive by RIBA™ 2.0 (Table III). No correlation was observed between HCV RNA status and pattern of antibody reactivity by RIBA 2.0. The mean titers of antibodies reactive with individual HCV antigens observed with these patients are shown in Figure 1a. In general, all antibody titers were lower in HCV RNA negative patients. Only one HCV RNA negative patient (no. 6) demonstrated antibody titers in the range of those patients who were HCV RNA positive. This patient also had the highest HAI score on the liver biopsy. Significantly, in this patient the period of risk of HCV exposure was relatively recent compared to other HCV RNA negative patients. Notably, anti-E2 titers were significantly lower for the HCV RNA negative patients (reciprocal titer 23 vs. 240, $P = .035$). Mean anti-c22-3 titer was also lower for the HCV RNA group, although this difference was not statistically significant. No difference in anti-c33c titers was observed between the two groups and anti-c100-3 titers were low in all patients.

We extended our analysis of anti-HCV titers to include specimens from eleven anti-HCV positive blood donors who presented with normal ALT levels at the time of

TABLE III. Pattern of Reactivity* of HCV Patient Serum to HCV Recombinant Antigens in SIA

Patient	511	c100-3	c33c	c22-3
HCV-RNA Negative				
1	0	0	1+	4+
2	3+	4+	4+	4+
3	0	0	2+	3+
4	3+	1+	4+	4+
5	1+	1+	0	4+
6	4+	4+	4+	4+
7	0	0	2+	3+
8	0	0	1+	4+
HCV-RNA Positive				
1	0	0	0	3+
2	1+	0	4+	4+
3	0	0	4+	4+
4	0	0	1+	4+
5	4+	4+	4+	4+

*Reactivity to HCV recombinant antigens was scored from 1+ (minimum) to 4+ (maximum) according to the manufacturer's recommendations. 0 = nonreactive

donation. All donor specimens were reactive by ELISA 2 and positive for anti-HCV by RIBA™ 2.0. Five were HCV RNA negative and six were HCV RNA positive. Mean antibody titers observed in these donor specimens are shown in Figure 1b. Differences in mean antibody titers between HCV RNA positive and negative donors were similar to that observed for the other anti-HCV reactive patients. Anti-E2 titers were significantly lower among HCV RNA negative donors (5 vs. 129, $P = .013$). Anti-c22-3 and anti-c33c titers were also lower (although not significantly) among HCV RNA negative donors.

DISCUSSION

Unlike previous studies, we have studied HCV RNA in both liver tissue and PBMCs in patients who were both HCV RNA positive as well as HCV RNA negative in serum. In only one previous report was HCV RNA measured in serum, PBMC, and liver [Romeo et al., 1993]. However, no anti-HCV reactive, serum HCV RNA negative patients were studied. Determination of HCV RNA in PBMCs is important because HCV is known to replicate in extrahepatic sites and it has been shown that absence of HCV RNA in liver at the conclusion of therapy does not preclude subsequent relapse of HCV, possibly due to an extrahepatic reservoir [Balart et al., 1993; Gurakar et al., 1995]. In the case of hepatitis B infection, persistence of HBV in PBMC has been demonstrated after apparent clearance of the virus [Michalak et al., 1994].

We noted absolute concordance between the status of HCV RNA in serum, liver, and PBMCs, in particular we did not find HCV RNA in either PBMCs or liver tissue when the serum HCV RNA was negative. Although the number of patients studied is somewhat limited, it would appear that the status of HCV RNA in the serum infers the presence or absence of HCV RNA in the liver, at least in patients with persistently normal ALT values recorded over a long period of time. This confirms the reported by Shindo et al. [1995b] who determined HCV RNA in the liver only, and Shakil et al. [1995] who performed HCV antigen staining in liver, which is less sensitive than the determination of hepatic HCV RNA. In patients with chronic hepatitis C infection, a similar concordance was noted in serum, PBMC [Romeo et al., 1994] and liver tissue [Romeo et al., 1994; Shindo et al., 1995a] in those who developed a complete and sustained response after interferon therapy. On the other hand, discordance of the status of HCV RNA between the serum and PBMC has been noted in untreated chronic hepatitis C patients with raised ALT values and in patients undergoing interferon therapy who had not demonstrated a sustained response [Zignego et al., 1992; Qian et al., 1992; Muratori et al., 1994].

Like others who have studied patients with persistently normal ALT values, we found mild necroinflammatory changes in patients who were HCV RNA positive [Alberti et al., 1992; Masafumi et al., 1994; Shindo et al., 1995b; Shakil et al., 1995]. A study by Alter et al. [1992] noted persistence of HCV RNA in the serum for up to 4 years after onset of acute illness in 15 patients with community acquired acute hepatitis C infection in whom ALT levels had normalized. This would suggest that spontaneous resolution of HCV infection seldom occurs. However, in our study, the anti-HCV reactive patients in whom serum, PBMCs, and liver HCV RNA were negative and whose liver histology was judged to be essentially normal, may represent individuals who have spontaneously cleared the infection.

Extensive serologic testing was then performed in an attempt to establish particular antibody profiles unique to viremic patients and patients who have resolved infection respectively. RIBA™ 2.0 antibody profiles did not differentiate active viremic HCV infection from nonviremic presumably resolved disease. However, ELISA testing using multiple HCV recombinant antigens demonstrated mean antibody titers to structural antigens, in particular HCV E2 antibodies, to be significantly lower in nonviremic patients.

Previous studies have indicated that HCV antibody titers frequently decline in individuals who appear to resolve HCV infection [Bonino et al., 1993]. However, attempts to identify specific serologic markers which correlate with disease status in HCV infected patients have been largely unsuccessful. Tanaka et al. [1991], using a first generation anti-HCV assay based on HCV c100-3 (NS4) recombinant protein, observed that a decline in anti-c100-3 titer was associated with a normalization of ALT in long term follow-up of twelve patients with post-transfusion hepatitis. However, these patients were not tested for HCV RNA. In our study of patients and donors who had been exposed to HCV and who presented with normal ALT levels, we found anti-c100-3 titers to be low in all cases, irrespective of their HCV RNA status. This finding corroborates recent findings by Barrera et al. [1995] who observed the loss of anti-c100-3 antibody in patients with self limiting hepatitis

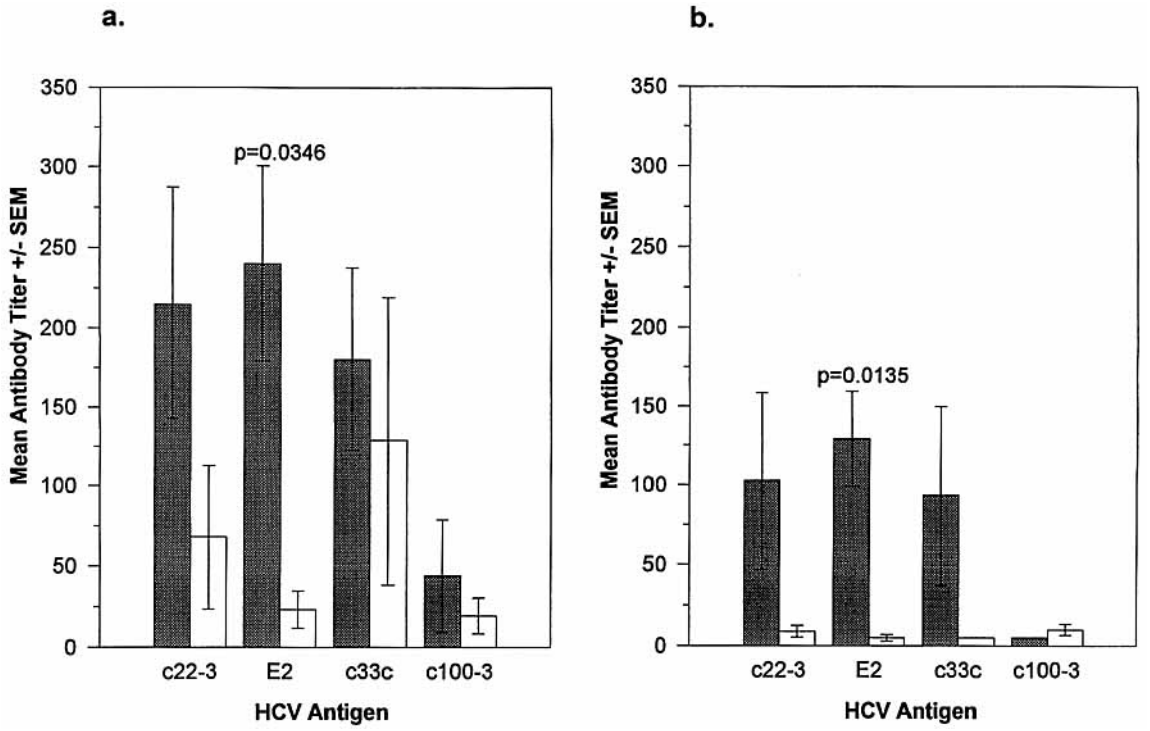

Fig. 1. Mean antibody titers (reciprocal) to HCV antigens in **(a)** HCV patients (■ RNA positive N = 5; □ RNA negative N = 8) and **(b)** anti-HCV positive donors (■ RNA Positive N = 6; □ RNA Negative N = 5). SEM = standard error of mean.

based on normalization of ALT levels, despite the presence of persistent or transient viremia.

More recent studies have suggested that measurements of antibodies to the structural core antigen of HCV may be useful in monitoring response to viral therapy in patients with chronic hepatitis C infection [Urushihara et al., 1994]. Our study supports this notion, since we found mean titers of antibodies to the structural core (c22-3) and envelope (E2) antigens to be substantially lower in both HCV RNA negative patients and donors. By contrast, no significant difference in mean titers of antibodies to the nonstructural antigens c33c (NS3) and c100-3 (NS4) were noted when HCV RNA positive and negative individuals were compared.

In particular, we found that mean anti-E2 titers were significantly lower in HCV RNA negative patients and donors. Recent studies have shown that mammalian derived envelope (E2) antigen is useful for the detection of anti-HCV in patients and blood donors [Zaaijer et al., 1994]. Moreover, antibodies to E2 have been shown to persist in patients with chronic infection [Chien et al., 1993; Zhang et al., 1995]. The N-terminal region of E2 has been shown to be hypervariable with regard to nucleotide and amino acid sequence [Weiner et al., 1991; Lesniewski et al., 1993] and it has been speculated that this region undergoes rapid mutation as a mechanism of escaping host immune response [Weiner et al., 1991]. Our results support the utility of anti-E2 titer as an indicator of ongoing viral replication in HCV infected patients. It is possible that anti-E2 production depends on ongoing immunogenic stimulation. Moreover our study suggests that longitudinal monitoring of anti-E2 titer may be a useful adjunct in the assessment of disease status, particularly in anti-HCV reactive patients who have persistent normal liver tests.

In summary, we found in our patients with persistently normal liver tests, that serum HCV RNA correlated absolutely with liver and PBMC HCV RNA. Patients with no detectable HCV RNA presumably have had spontaneous resolution of their HCV infection. Also, the patients we studied, even those who were RNA positive, had only mild histological changes in the liver. Additionally, anti-E2 titer appears to be a useful marker of ongoing viral replication.

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